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PREPARATION AND QUANTIFICATION OF ENTOMOPHILOUS POLLEN USING SONICATION AND AN AREA-COUNTING TECHNIQUE

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Abstract

Large pollen grains are difficult to quantify accurately. In order to ensure none have escaped, the anther must be removed and preserved before it is fully mature, leaving the pollen grains somewhat fragile and cohesive. Any method of quantification requires that they be separated into discrete grains, a difficult process, in part, due to their immature state. A probe sonicator can effectively disperse pollen grains from an opened anther in a petri dish partially filled with water. If the grains are too large to readily remain in suspension they can be allowed to settle in the petri dish and then quantified. As long as the grains are uniformly distributed in the petri dish, a compound microscope can be used to count the pollen grains in a field of view, and the contents of multiple fields of view can be used to extrapolate the total number of pollen grains.

Key Words: Sonication, pollen counting, entomophilous, *Calochortus*.

When studying reproductive biology, pollen counts are important because pollen/ovule ratio is an essential aspect of plant breeding systems (Cruden 1977). Methodology for the preparation and counting of entomophilous pollen grains from indehisced anthers is complicated by several factors. In order to get accurate counts in terms of grains per anther, the sample anther must be indehisced to ensure all pollen is present. Collecting young anthers often means the pollen grains are less developed, softer, and more fragile, thereby making complete grain removal from the anther more difficult. Furthermore, entomophilous pollen grains tend to be fairly sticky and may readily adhere to each other even once separated. Sonication, the process of passing sound waves through an aqueous medium, can be effective at separating pollen grains. Cohesion can be easily avoided with the addition of a surfactant (i.e., Trition X-100); however, the vibration produced by sonication causes excessive foaming, rendering the pollen difficult to view when a surfactant is added. Besides its cohesive properties, entomophilous pollen is difficult to count because it tends to be too large for a coulter counter and may be challenging to suspend in solution well enough to use a hemacytometer.

B. E. Vaissiere (1991, A. Dafni 1992) developed a protocol for pollen sonication using acetone as the solvent and a sonicator with a horn. However, this technique requires the sample to sit in acetone for thirty minutes prior to sonication. An alternative technique for sonication has been developed (Kannely 2003). By using distilled water instead of acetone and a sonicator with a probe, the same results can be achieved in less time since the pollen does not need to sit in the water prior to sonication.

Protocol for Sonication of Pollen

Prepare the sample by placing a single indehisced anther in a petri dish filled halfway with distilled water. The anther can either be fresh or preserved in a mixture of formalin, acetic acid and alcohol (FAA), a common botanical preservative. Then using forceps and a needle pull the anther sacs apart. Fix the sonicator'sprobe to a stationary stand and lift the petri dish until the probe is two thirds of the way to the bottom of the dish from the surface of the water. A model VC 50 Vibra Cell sonicator set at 20 μm amplitude worked for a variety of *Calochortus* species (Liliaceae). The dish should then be moved around by hand to cause equal vibration throughout the entire area. The dish should also be raised and lowered slightly, which will cause the broken anther to move towards the probe and to break apart more. It is important to move the dish in straight lines, rather than in circles, as the latter will cause a current in the round dish that would prevent uniform settling of the pollen grains. Pieces of anther float in the suspension after sonication, and when lifted out with fine forceps and examined under a dissecting microscope, can be seen to be devoid of pollen.

Sonication between 12 and 15 seconds was adequate for most *Calochortus* species, depending on the maturity of the pollen grains. Maturity of pollen grains can be determined in the field by subjective comparisons between mature and immature filaments, anthers and pollen grains for a given species. Since most species ultimately reach different sizes it is best to sonicate anthers with a varying degree of maturity in order to find the optimal point. Less mature pollen grains can be mechanically damaged by sonication, but fully developed pollen grains spend a very short time in indehisced anthers. The amount of time mature pollen grains will remain in indehisced anthers varies according to species and weather conditions, as warm dry weather can increase the rate of flowering phenology.

After sonication, the pieces of anther remaining can be easily removed; as they will be floating on the water at the top since they have a low density. The empty anther can be removed with forceps and examined with a dissecting microscope to ensure all the pollen grains were removed. The petri dish should immediately be set where counting will be done so the pollen can settle in a single layer at the bottom of the dish.

Counting Pollen (by Area)

Techniques for counting pollen vary in part based on grain size and number of grains produced per anther. Generally, entomophilous pollen is too large and heavy to count suspended in solution (with a hemacytometer or a coulter counter), and too numerous to count all individual grains. Jokerst (1980) counted pollen grains in a variety of *Calochortus* species with a hemacytometer. However, I found that Calochortus grains were too heavy in suspension and fell disproportionately in the channels of the hemacytometer. Kearns and Inouye (1993) describe a variety of general techniques for quantifying pollen. They only briefly mention using a microscope to scan a field of view. However, entomophilous pollen that has settled out of solution uniformly can be quantified by counting the number of pollen grains in a specified area. Ideally, the best technique would be to take a picture of all the pollen grains in the entire petri dish and quantify them using a computer program. Otherwise, a small subset of the total pollen, such as four fields of view at medium power (about 100×) under a compound microscope, can be counted and used to find the total number of pollen grains in the sample. Most entomophilous pollen grains settle to the bottom of a petri dish half filled with water in a matter of seconds whereas the rest of the particulate organic matter (remaining anther tissue) in the dish tends to float. Thus, it is possible to determine the number of pollen grains in the petri dish multiplying the number of pollen grains in the sample area by the fraction of the entire area of the dish (Fig. 1) that the sample area represents.

The field of view is the area viewed under medium power (100×) with a compound microscope; it is calculated after measuring the diameter with a stage micrometer. Then the diameter of the dish can be measured with calipers and its area calculated. A petri dish with walls perpendicular to the bottom must be used so area can be determined accurately. Care should be taken to use the same petri dish every time to ensure a consistent area measurement. For each sample (i.e., the contents of one anther), the number of pollen grains in four fields of view should be counted, one from each quadrant of the petri dish. Grains partially in view should be counted on the left half of the field of view and not counted on the right half. The totals can then be summed and used to determine the total number of pollen grains in the entire dish (i.e., in one anther). Each anther can be sampled, or one anther per flower can be used to determine the total number of pollen grains in the flower. Dish area and field of view area can be calculated as follows:

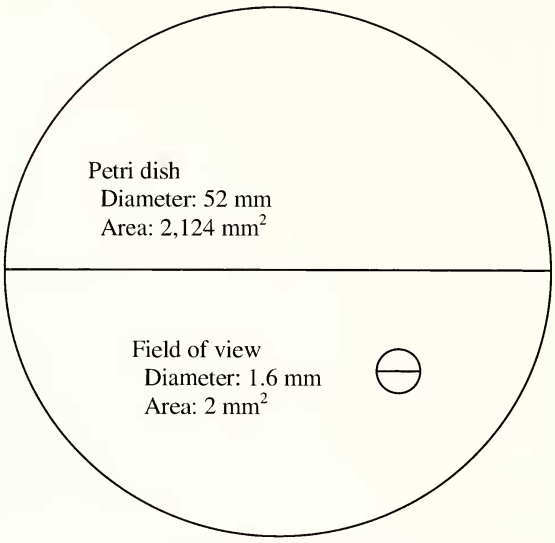

Fig. 1. Pollen Counting by Area (Πr^2), using petri dish and field of view diameters (not to scale).

$$\text{Pollen grains per dish} = \frac{(\text{total grains per fields of view counted})(\text{petri dish area})}{(\text{total area of fields of view counted})}$$

$$\text{Pollen grains per flower} = (\text{grains per dish})(\text{number of stamens})$$

Estimation of Accuracy

Triteleia ixiodies ssp. *analina* (collected in Butte County, CA) was chosen to make total pollen counts because while it has entomophilous grains similar to *Calochortus* spp. it has a relatively low number of grains per anther (Schlising, unpublished). Total pollen grains per anther on three separate plants were counted with a dissecting microscope, resulting in counts of; 1620, 2228 and 1527, with an average of 1,792. In comparison, 24 plants of *T. ixoides* were sampled using the field of view estimation technique previously described. This count ($X \pm SE$) was 10 ± 0.8 per four fields counted, and the estimate for total grains per anther was 2158 ± 170 (range 1511–3670; $n = 24$).

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